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ANALYTICAL METHOD DEVELOPEMTNET AND VALIDATION FOR SIMULATANEOUS DETERMINATION OF DIOSMIN AND HESPERETIN IN ORANGE PEEL CITRUS AURANTIUM BY RP-HPLC

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Abstract: A fast, simple, correct and robust reversed phase HPLC technique for the simultaneous determination of two flavonoids, hesperidin and diosmin, in combined drugs became developed and confirmed. This technique uses a quick C18 Silica Column with dimentions 4.6 x 250 mm with 5µ column particles thermostated at 25 °C, and a cell segmentcomposed of Methanol: 1% OPA in Water, introduced at a waft fee of 1.0 ml/min, with UV detector sign tracking at 280 nm and an injection volume of 20 µl. those chromatographic conditions yielded chromatograms with symmetric peaks of hesperidin, eluting at a 2 min retention time, and diosmin, at equal to 4.five min retention time, with a total run time cycleof 35 min. The approach validation parameters confirmed top notch values for accuracy, linearity and reproducibility. This method is suitable for routine evaluation in pharmaceuticaland food pleasant control laboratories.

Key Words: RP-HPLC, Method Development, hesperidin, diosmin, Column Etc.

INTRODUCTION:

Diosmin and hesperidin are molecules that belong to a set of natural compounds known as flavonoids. Flavonoids are a big organization of plant pigments sharing the same primary chemical structure ture, i.e. a three-ringed molecule with hydroxyl (OHcompanies) attached. Diosmin (C28H32O15) (Fig. 1a) is a obviously occurring flavonoid glycoside that may be remoted from various plant sources or derived from the flavonoid hesperidin. It changed into first remoted in 1900 from Scrophularia nodosa, and

primary brought as a healing agent in 1969. Diosmin is considered to be avascular-protective agent used to treat continual venous insufficiency, hemorrhoids, lymphedema and varicose veins. As a flavonoid, diosmin also exhibits, freeradical scavenging and antimutagenic houses. It differs molecularly from hesperidin with the aid of the presence of a double bond between the carbon atoms within the critical carbon ring. Diosmin can be synthetic by way of extracting hesperidin from citrus rinds, observed through the conversion of hesperidin to diosmin. Diosmin has been used for extra than 30 years as a phlebotonic and vascular-shielding agent, and has recently started to be investigated for different therapeutic functions, together with most cancers, premenstrual syndrome, colitis and diabetes. Hesperidin is the essential flavonoid in citrus culmination, commonly in candy orange and lemon, and consequently in juices made of those citruses. This mixture of flavonoid tablets has been used to manipulate the signs and symptoms of persistent venous disorder, inclusive of edema of the decrease limbs, for decades.[1-6]

METERIALS AND METHODS:[7-11]

Procurement and processing of plant raw materialCollection of Orange Peels

Orange peels was procured from Sami-Sabnisa group, Karnataka, India.Raw material was ground into coarse powder using grinding mill.

Extraction process: The dried Orange peels was (500 g) powdered mechanically using commercial electrical stainless steel blender and extracted with ethanol, ethyl acetate, methanol, ethanol ; water (50;50 v/v) , methanol ; water (50;50 v/v) and water in a round bottom flask reflux method separately until exhaustion at temperature near respective solvents boiling point and the extract was concentrated under Reduced pressure 22–26 mg/Hg at 45°C, and the residue obtained was stored at 5°C.

Solubility studies: Solubility was performed as per USP guidelines.

Table No. 1: Standard drug sample supplier and manufacturer.

sr.no.	Name of drug	Supplier name
1	Diosmin	yuccaenterprises@yahoo.com
2	Hesperetin	yuccaenterprises@yahoo.com

HPLC method development:

Preparation of standard stock solution:

1. Diosmin: Accurately weigh and transfer 10 mg standard of Diosmin into 100 ml of volumetric flask and dissolved in methanol, make the volume up to 100 ml with methanol to obtain stock solution.

2. Hesperetin: Accurately weigh and transfer 10 mg standard of Hesperetin into 100 ml of volumetric flask and dissolved in methanol, make the volume up to 100 ml withmethanol to obtain stock solution.

Selection of mobile phase:

Diosmin and Hesperetin was injected in to the HPLC system and run in different solvent system matrix of different solvent were tried in order to determine optimum chromatographic conditions for effective separation after several permutation and combination, it was found that mixture of Methanol gradient : 0.1 % orthophosphoricacid in water gives satisfactory result as compared to other mobile phase with gradient programme, as it gave high resolution of Diosmin and Hesperetin with minimal tailing.

Preparation of mobile phase:

Final, optimal composition of mobile phase contain gradient programme of methanolgradient and 0.1 % orthophosphoric acid in water.

Chromatographic condition:

Following are the optimized chromatographic condition for RP-HPLC method.

Table No. 2: Optimized chromatographic condition

Parameter	**Values**
Column	C18 Silica Column Id; 4.6 x 250 mm 5µ
Wavelength	280 nm
Flow rate	1.0 ml/min
Injection volume	20µl
Temperature	25ºC
Run time	35 minutes

Assay of Drug:

Approximately 10 mg of each Diosmin and Hesperetin were accurately weighed. Transfer to separate 10 ml volumetric flask. Dissolved in the methanol and dilute to the volume same solvent mixture to furnish stock solution containing 1000µg/ml of Diosmin and Hesperetin from this solution appropriate dilution of standards were made to get the final concentration and finally the solution were filtered through the what man filter paper. A 20 µl sample was injected under optimized chromatographic condition. The peak were measured at 280 nm and the percent purity and % RSD wascalculated.

System suitability test:

System suitability is a pharmaceutical requirement and is used to verify, whether the resolution and reproducibility of chromatographic system are adequate for analysis to be done Acceptance criteria

- RSD should not be more than 2.0 % for five replicate injections of standard.
- USP tailing factor is not more than 2.0
- The column efficiency is determined as a no. of theoretical plates should be morethan 3500 for diosmin and more than 8000 for hesperetin.

Validation of the developed RP-HPLC method:

The developed method was validated as per ICH guideline for its system suitability, linearity, accuracy, precision, robustness, limit detection, limit of quantification by using following procedure.

Initialization of instrument: The HPLC instrument was switched on. The column was placed in the instrument and washed and then saturated the column with the mobile phase 30-40 minutes. Filed the HPLC vial with the diluents.

Specificity:

Specificity is the ability to measure accurately and specifically the analyte of interestin the other component that may be expected to be present in the sample matrix.

Distinguish an analyte from known impurities, synthetic precursors, metabolite or degradation product and other inactive. It should be shown by its resolution with these compounds. Check the interference of placebo by injecting in to the equilibrated system. These should not be any peak at the R.T. of main peak. Acceptance criteria;It should pass the peak purity criteria.

Linearity:

Linearity of an analytical method is an analytical method is its ability to elicit test result that are directly or by a well-defined mathematical transformation proportionalin the concentration of analyte in sample within a given range.

Accuracy:

The accuracy of analytical method is the closeness of the test results obtained by thatmethod to the true value. It is measure of the exactness of the analytical method developed. Accuracy may often be expressed as percent recovery by the assay of a known amount of analyte added.

Determination: The accuracy is calculated from the test result as the percentage of analyte recovered by the assay.

Acceptance criteria: The relative standard deviation should not be more than 2.0%

Precision:

Precision of an analytical method is the degree of agreement among individual test result when the procedure is applied repeatedly to multiple sampling of a homogenous sample.

Determination:

a. Repeatability Standard solution of Diosmin and Hesperetin were prepared andchromatogram is recorded. Area was measured of the same concentra- tion solution three times and %RSD was calculated.

b. Intraday precision: standard mixture solution containing Diosmin and Hesperetin analyzed three times on the same day and % RSD was calculated.

c. Interday precision: Mixed solution containing Diosmin and Hesperetin analyzed three times on different day and %RSD was calculated. Acceptance criteria: The relative standard deviation should not be more than 2% for test result.

LOD and LOQ:

LOD and LOQ were calculated from the average slope and standard deviation from the cali- bration curve as per ICH guideline.LOD=3.3 X SD/S

LOQ=10 X SD/S

Robustness:

It is measure of capacity of the method to remain unaffected by small but deliberate variation in method parameter and provides an indication of its reliability under normalusage Determination: The robustness of an analytical method is determined by analysis of aliquots from homogenous lots by differing physical parameter that may differ but are still within the specified parameters of the assay. For example change in physical parameter like PH of mobile phase and its ratio. Standard preparation, placebo preparation and sample preparation in triplicate were prepared. The sample along withstandard and placebo were injected under different chromatographic condition such asChange in flow rate (±0.1 ml/min), Change in wavelength (± 2nm).

RESULTS AND DISCUSSION:

Figure 1: *citrus aurantium* Orange peel - coarsely ground in GrindingMill

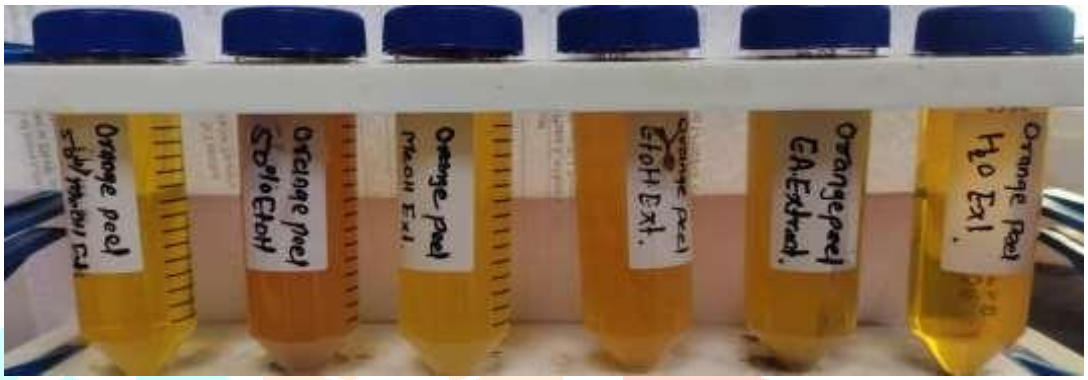

Figure 2: *citrus aurantium* Orange peel extracts in 5 various solventsystems

Figure 3: *citrus aurantium* Orange peel Ethanol-Water (50;50 v/v) dried extracts

Percentage yield of various Extracts of Orange peel *citrus aurantium*

Table 3: Percentage yield of various Extracts of Orange peel *citrus aurantium*.

Temperature (± 5ºC)	Name of Solvent	Percentage Yield
78ºC	Ethanol	11.42
65ºC	Methanol	11.09
89ºC	50 % Ethanol	14.45
83ºC	50 % Methanol	13.43
77ºC	Ethyl Acetate	7.89
95ºC	Water	1.93

Result of phytochemical analysis of flavonoids

Table 4: Phytochemical analysis of flavonoids

Test	Remark	Inference
Test for Flavonoids Shinoda test	Orange pink colour Fl observed	avonoids are present

Solubility Study:

These studies were carried out to find an ideal solvent in which drug is completely soluble. Various solvents were tried for checking solubility of diosmin and hesperetin. From solubility studies it was concluded that flavonoids are freely soluble in Methanol.Hence, for further study was Methanol used as solvent.

TLC and HPTLC

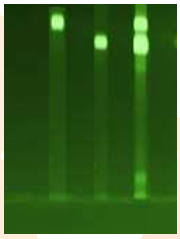

Figure 5: TLC of standards and extract of Orange peel citrus aurantium

Here, 1- Diosmin, 2- Hesperetin, 3- Ethanolic extract of Orange peel.

After several permutations and combinations for the purpose of optimisation best separation was observed in mobile phase having mixture of Tolune-ethyl acetate-formic acid in ratio 5:4:1. So same was applied for HPTLC.

Trial: In this trial changes are made of chromatographic condition such as Mobile phase ratioas from Vendor Method**.**

Trial 3

Table 5: Chromatographic conditions of trial 3

Parameter	Condition
Column	Inertsil 4.5 x 250mm 5µ C18 silica
Mobile Phase	Methanol : 1% OPA in Water
Flow rate	1 ml /min
Run Time	35 min
Column Temperature	Ambient
Injection Volume	20µl
Detection wavelength	280nm
Diluent	Methanol

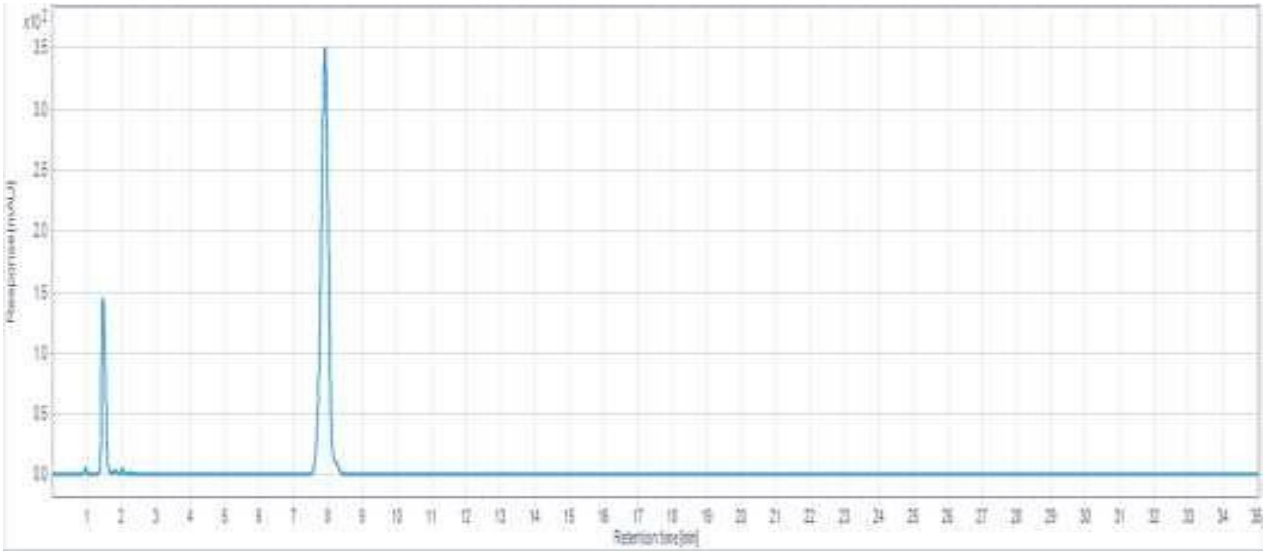

Figure 6: Chromatogram of Diosmin and Hesperetin trial 3.

Conclusion: Peak appeared with no blank interference and peak shapes weresatisfactory

Validation of developed method.

Percent assay of Ethanol extract of Orange peels

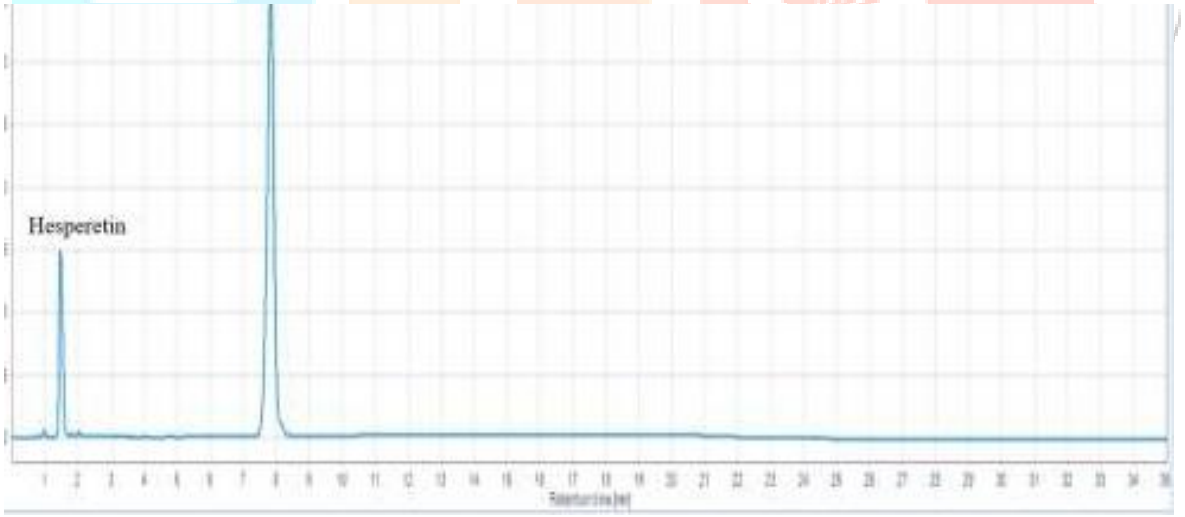

Figure 7: Chromatogram of sample extract

Table 6: Percentage assay of Ethanol extract

Sr.no.	Name ofDrug	Wt. of Sample (gm)	Sampledilution(ml)	Wt. of standard(gm)	Area ofStardad	Sample Area	% Content (%w/w)
1	Hesperetin	**0.**1391	**50**	0.0524	303671	310154	27..9
2	Diosmin	**0.**1032	**50**	0.0565	709832	692602	65.3

Specificity and system suitability

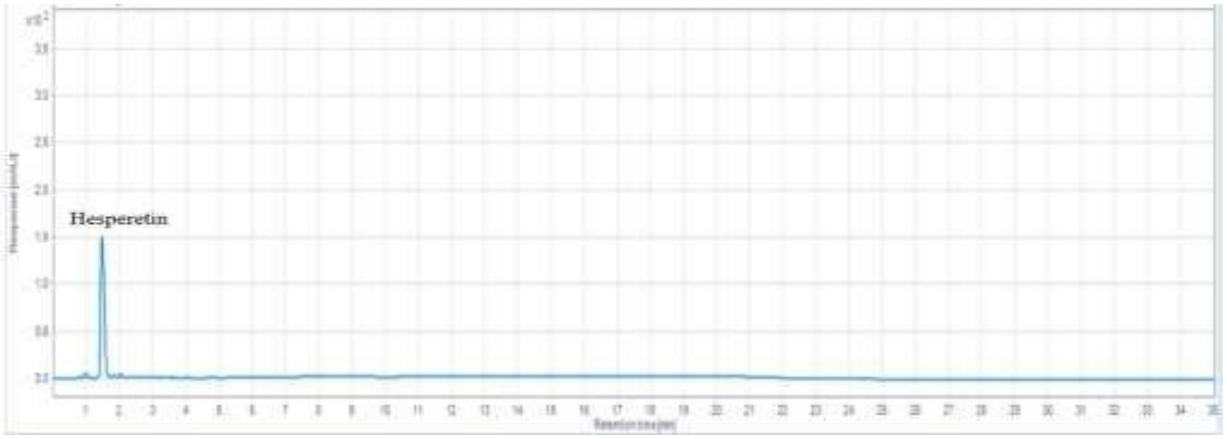

Figure 8: Specificity chromatogram of Diosmin standard

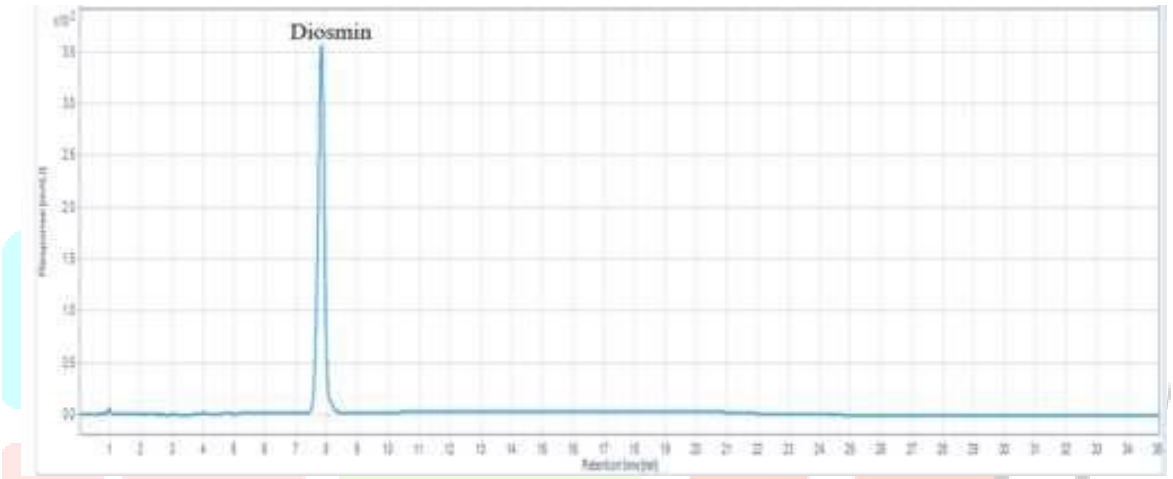

Figure 9: Specijficity chromatogram of Hesperetin standard For Hespereti

No. of Injection	Retention time	Area	Theoretical plates (USP)	Tailing factor (USP)
1	1.53	307648	2639	1.21
2	1.52	303671	2693	1.20
3	1.52	308470	2627	1.21
4	1.52	306721	2654	1.19
5	1.52	307136	2681	1.20
6	1.52	307845	2690	1.22
AVERAGE	1.52	307564	2664	1.20
STD DEV	0.004	670.94	27.92	0.010
% RSD	0.268	0.21	1.04	0.87

Table 7: Specificity data of Hesperetin Standard Conclusion:

Sr. No.	Parameter	Result	Limit
1	There is no interference from blank at the retention time of Hesperetin peak	Complies	Complies
2	% RSD of retention time	0.268	NMT 2.0%
3	% RSD of area	0.21	NMT 2.0%
4	Theoretical plates	2664	NLT2000
5	USP tailing factor of Hesperetin peak	1.20	NLT 1.0

Table 8: Conclusion of Specificity data of Hesperetin

For Diosmin

No. of Injections	Retention time	Area	T. plates (USP)	T.factor (USP)
1	7.92	709832	2379	1.14
2	7.91	709673	2376	1.13
3	7.91	706398	2307	1.13
4	7.91	704095	2359	1.13
5	7.92	708250	2341	1.14
6	7.91	706938	2310	1.13
AVERAGE	7.91	707531	2345	1.13
STD DEV	0.005	2183	31.61	0.005
%RSD	0.06	0.30	1.34	0.45

Table 9: Specificity data of Diosmin Standard

Conclusion :

Sr. No.	Parameter	Results	Limit
1	There is no interference from blank at the retention time of Diosmin peak	Complies	Complies
2	% RSD of retention time	0.06	NMT 2.0%
3	% RSD of area	0.30	NMT 2.0%

4	Theoretical plates	2345	NLT2000
5	USP tailing factor of Diosmin peak	1.13	NLT 1.0

Table 10: Conclusion of Specificity data of Diosmin

Method Precision

For Hesperetin

No. of Injections	**Retention time**	**Area**	**T. plates (USP)**	**T. factor(USP)**
1	1.53	307648	2639	1.21
2	1.52	303671	2693	1.20
3	1.52	308470	2627	1.21
4	1.52	306721	2654	1.19
5	1.52	307136	2681	1.20
6	1.52	307845	2690	1.22
AVERAGE	1.52	307564	2664	1.20
STD DEV	0.004	670.94	27.92	0.010
% RSD	0.268	0.21	1.04	0.87

Table 11: Precision data of Hesperetin Standard

Conclusion:

Sr	**Wt of Sample(gm**	**Sample dilution(ml)**	**Wt. of standard(gm)**	**Sample Area**	**% Content(w/w)**
1	0.1139	50	0.05	29983	28.43
2	0.1046	50	0.05	29464	28.12
3	0.1274	50	0.05	29787	28.71
4	0.1950	50	0.05	29413	28.49
5	0.1834	50	0.06	29096	28.58
AVERAGE	–	–	–	–	28.48
STD DEV	–	–	–	–	0.19
% RSD	–	–	–	–	0.69

Table 12: Conclusion of Precision data of Hespereti

For Diosmin

No. of Injections	Retention time	Area	Theoretical plates (USP	Tailing factor (USP)
1	7.92	70983	2379	1.14
2	7.91	709673	2376	1.13
3	7.91	706398	2307	1.13
4	7.91	704095	2359	1.13
5	7.92	708250	2341	1.14
6	7.91	706938	2310	1.13
AVERAGE	7.91	707531	2345	1.13
STD DEV	0.005	2183	31.61	0.005
%RSD	0.06	0.30	1.34	0.45

Table 13: Precision data of Diosmin Standard

Conclusion:

Sr.no	Wt of Sample (gm)	Sample dilution(ml)	Wt. of Standard (gm)	Sample Area	% Contet (%w/w)
1	0.1173	50	0.05	704633	63.16
2	0.1078	50	0.05	705308	63.05
3	0.1576	50	0.05	709385	63.17
4	0.1423	50	0.05	708250	63.01
5	0.1432	50	0.05	706912	62.97
AVERAGE	_	_	_	_	63.08
STD DEV	_	_	_	_	0.08
% RSD	_	_	_	_	0.13

Table 14: Conclusion of Precision data of Diosmin

Linearity:

For the construction of calibration curves, five calibration standard solutions were prepared over the concentration range. The linearity was determined for Diosmin and Hesperetin in the range of 10-50 µg/ml. The correlation coefficient values of 0.999. The linearity results are shown in table.

Number of Injections	Hesperetin	Diosmin
1	249375	657025
2	246019	652342
3	248597	657472
4	249325	650287
5	246510	653629
6	247284	657383
Average	247851	654689
Standard Deviation	1451	3048
% RSD	0.58	0.46

Conclusion

Concentration	Area of Hesperetin	Area of Diosmin
10	294723	772531
20	598780	1545162
30	899724	2317893
40	1195431	3093024
50	1477207	3862455
R2	0.999	0.999
Intercept	468323+25184	1261664+27372
Slope	25184	27372

Table 15: Conclusion of Linearity data

LOD and LOQ of Diosmin and Hesperetin

Parameter	Hesperetin (µg/ml)	Diosmin (µg/ml)
LOD	0.82	0.23
LOQ	2.92	3.64

Table 16: Result of LOD and LOQ for Diosmin and Hesperetin

Range

1. **Range Lower 50%**

Table 17:Range at 50% level

Table 18: Range at 150% level

Number of Injections	Hesperetin	Diosmin
1	349232	818973
2	343496	817340
3	342471	812387
4	345947	818424
5	343620	814589
6	341893	815635
Average	344443	816224
Standard Deviation	2726	2505
%RSD	0.79	0.30

Result:

Table 19: Conclusion of Range 50% and 150%

Sr. No.	Parameter	Hesperetin	Diosmin	Limit
1	% RSD of Area in Range 50%	0.58	0.46	NMT 2%
2	% RSD of Area in Range 150%	0.79	0.30	NMT 2%

CONCLUSION:

In the present research work, a successful attempt was made for Development and Validation of RP-HPLC methods for estimation of Diosmin and Hesperetin. The method developed for analysis of Diosmin and Hesperetin is simple, rapid, precise, and economical. The method was validated and satisfactory results were obtained for some of the characteristics tested. The system suitability parameters were selected for RP- HPLC method and has data shown good peak intensity, good retention time, good Asymmetry of the drug that define the suitability method. It can be easily and conveniently adopted for routine quality control analysis. All methods are simple, rapid,precise and economic. So, method can be used for the routine analyst Determination of Diosmin and Hesperetin in bulk and herbal dosage form.

REFERENCES:

1. Diosmin, monograph, Alternative Medicine Review, 9 (3), 308–311 (2004).
2. V. Kuntić, J. Brborić, I. Antunović-Holclajtner, S. Uskoković-Marković, Evaluating the bioactive effects of flavonoid hespeidin – A new literature data survey, Vojnosanitetski pregled,71 (1), 60–65 (2014). DOI: 10.2298/VSP1401060K.
3. P. Gloviczki (ed.), ass. eds. C. M. Dalsing, B. Eklof, L. G. Moneta, W. T. Wakefield, Handbook of Venous Disorders, 3rd ed., Guidelines of the American Venous Forum, CRC Press, Taylor &Francis Group, LLC, 2008.
4. D. Srilatha, M. Nasare, B. Nagasandhya, V. Prasad, P. Diwan, Development and validation of UV spectrophotometric method for simultaneous estimation of hesperidin and diosmin in the pharmaceutical dosage form, International Scholarly Research Notices (ISRN) Spectroscopy 2013, 4 pages. DOI: 10.1155/2013/534830.
5. Z. Moldovan, A. A. Bunaciu, A. M. Al-Omar, Y. H. Aboul-Enein, A spectrophotometric method for diosmin determination, The Open Chemical and Biomedical Methods Journal, 3, 123–127 (2010).
6. Feng CH, Otani C, Ogawa Y. Innovatively identifying naringin and hesperidin by using terahertz spectroscopy and evaluating flavonoids extracts from waste orange peels by coupling with multivariate analysis. Food Control. 2022 Jul 1;137:108897.
7. Ozturk B, Parkinson C, Gonzalez-Miquel M. Extraction of polyphenolic antioxidants from orange peel waste using deep eutectic solvents. Separation and Purification Technology. 2018 Nov 29;206:1-3.
8. Tsouko E, Maina S, Ladakis D, Kookos IK, Koutinas A. Integrated biorefinery development for the extraction of value-added components and bacterial cellulose production from orange peel waste streams. Renewable Energy. 2020 Nov 1;160:944-54.
9. Zafar M, Arshad F, Faizi S, Anwar Z, Imran M, Mehmood RT. HPLC based characterization of citric acid produced from indigenous fungal strain through single and Co-Culture fermentation. Biocatalysis and Agricultural Biotechnology. 2020 Oct 1;29:101796.
10. Dumbravă DG, Hădărugă NG, Hădărugă DI, Moldovan C, Raba D. Determination by RP-HPLC of β-carotene concentration from orange (Citrus sinensis L.) fruits peel extracts. Journal of Agroalimentary Processes and Technologies. 2010;16(2):242-6.
11. Xu Y, Lv X, Yang G, Zhan J, Li M, Long T, Ho CT, Li S. Simultaneous separation of six pure polymethoxyflavones from sweet orange peel extract by high performance counter current chromatography. Food chemistry. 2019 Sep 15;292:160-5.